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Reproductive and Multigenerational Effects of Dietary Benzo[A]Pyrene Exposure in Zebrafish

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REPRODUCTIVE AND MULTIGENERATIONAL EFFECTS OF DIETARY
BENZO[A]PYRENE EXPOSURE IN ZEBRAFISH

by
Mallory Beatrice White

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of
the requirements of the Sally McDonnell Barksdale Honors College.

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May 2014

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ABSTRACT

Reproductive and Multigenerational Effects of Dietary Benzo[a]pyrene Exposure in Zebrafish

Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) that is a known carcinogen that also acts as an endocrine disruptor. Multigenerational impacts of PAH exposure are suggested in human cohort studies. To investigate mechanisms and developmental phenotypes associated with a dietary BaP exposure, zebrafish (*Danio rerio*) were used. We hypothesized that BaP exposure would cause: 1) phenotypic malformations in the gonad that would affect reproductive success; 2) developmental deformities in the offspring that would lead to decreased survival and ability to reproduce without additional BaP exposure; and 3) changes in gene expression that would be conserved across generations and related to the adverse phenotypes observed. Adult zebrafish (2 females x 2 males, N=10 replicate tanks per treatment) were fed 2% body weight/day flake food treated with 0, 10, 114, or 1012 μg BaP/g flake (equivalent to 0, 0.21, 2.3, and 20 μg BaP/g fish/day) for 22 days. Parental gonads were sectioned and evaluated for pre-vitellogenic, vitellogenic, mature, and corpus atreticum oocytes in females and spermatogonia, spermatocytes, spermatids, and spermatozoa in males. Ovarian atresia was significantly decreased following high dose BaP exposure. The number of fertilized eggs was significantly decreased in F0 fish exposed to 20 BaP/g fish. Total egg production was not significantly affected by dietary exposure to BaP in the F0, F1, and F2 generations. Three subsequent untreated generations of offspring (F1-F3) were assessed for mortality and time to hatch at 24, 32, 48, 56, 72, 80, and 96 hours post

fertilization (hpf) and developmental deformities at 96 hpf. F1 (but not F2 or F3) mortality was significantly increased in larvae whose parents were exposed to 2.3 and 20 µg BaP/g fish by 48 and 56 hpf, respectively. Time to hatch in the higher doses significantly decreased in only the F1 generation. F1 body length, body shape, and brain shape were negatively impacted by parental exposure to 2.3 and 20 µg BaP/g fish. Molecular analysis is now being used to elucidate mechanisms that are associated with the phenotypic deformities detected across generations. Supported by NIEHS R21ES019940.

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INTRODUCTION

1.1 Benzo[a]pyrene

Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) that is an environmentally relevant carcinogenic and endocrine disrupting compound that causes multigenerational effects in vertebrates (Figure 1). PAHs generally occur as mixtures such as in cigarette smoke rather than single compounds. BaP and other PAHs are products of combustion from sources such as wildfires, industrial processes, automobile exhaust, energy production and use, food preparation, smoking tobacco, and open trash burning. BaP and its metabolites can bind to DNA and cause mutations that can lead to cancer. BaP is also suspected to affect reproduction and fertility.

1.2 Benzo[a]pyrene and diet

Exposure to BaP and other PAHs occurs by way of inhalation of atmospheric pollution from things such as cigarette smoke, forest fires, industrial emissions, and vehicle exhaust (Suzuki and Yoshinaga 2007, Inyang et al. 2003). For non-smoking humans, however, dietary intake accounts for the largest source of exposure (Suzuki and Yoshinaga 2007). Some of the major sources of BaP in the diet include processed and cured meat, shellfish, sea food products, and cereals (Duarte-Salles et al. 2013; Panel on Contaminants in the Food Chain, 2008). Exposure concentrations vary based on cultural diet and methods of food preparation. For example, estimated mean dietary exposure for Japanese, Norwegian, and European cultures of BaP was reported to be 91 ng/day, 149

ng/day, and 235 ng/day, respectively (Suzuki and Yoshinaga 2007, Duarte-Salles et al. 2013, and Panel on Contaminants in the Food Chain, 2008).

1.3 Parental benzo[a]pyrene exposure and fetal health

Maternal diet is one of the largest sources of fetal exposure to toxins such as BaP. Studies show that there is a significant connection between intake of BaP in the diet during pregnancy and decreased birth weight and length in humans (Duarte-Salles et al. 2013). Other studies in which exposure to BaP was airborne show that prenatal exposure to PAHs like BaP can significantly reduce birth weight, length, and head circumference in the offspring of those exposed (Choi et al. 2006). Prenatal airborne exposure to PAHs has a negative impact on cognitive development comparable to low-level lead exposure in offspring of exposed mothers (Perera et al. 2006). Follow up research showed decreased full-scale and verbal IQ scores in these children (Perera et al. 2009).

1.4 Benzo[a]pyrene exposure and reproductive health

There is evidence that BaP, and other PAHs, have a negative impact on female fertility and ovarian function. In mice, BaP has caused premature follicular activation and atresia in the ovaries of exposed females as well as a reduction in oocyte-sperm fusion in the process of fertilization (Sobinoff et al. 2012). BaP also has negative effects on male fertility. Exposure to BaP reduced motility of sperm in male rats following inhalation exposures (Inyang et al. 2003). Male rats exposed to BaP orally also experienced testicular atrophy, reduced epididymal sperm count, reduced sperm motility, and reduced sperm fertilization ability (Mohamed et al. 2010). Mohamed et al. also found

multigenerational reduction of these characteristics in subsequent F1 and F2 generations but no significant effects on the F3 generation (2010).

1.5 Zebrafish as a model

Danio rerio, or zebrafish, is a small tropical vertebrate fish that is recognized as a good model for human disease, particularly in studies of development. Zebrafish are a desirable model because they have a high instance of genetic similarity to humans (Howe et al. 2013), reproduce quickly with large numbers of eggs, have transparent embryos that are easily studied and examined throughout development, and are easy and inexpensive to raise and maintain (Santoriello & Zon, 2012). This non-mammalian model is becoming more common in studies examining developmental deformities. Zebrafish exposed to PAHs had reduction in length as well as curvature of the trunk and tail (Incardona et al. 2004). These developmental phenotypes can be used to investigate cellular mechanisms responsible for human developmental deformities due to exposure to PAHs such as BaP.

1.5 Goal of this study

An adverse outcome pathway is a conceptual model that links a direct molecular initiating event and an adverse outcome at a biological level of organization. Studies of problems that arise after human BaP exposure have proven it to be harmful to offspring and development. We hoped to use this study to determine the specific effects of parental dietary exposure to BaP on reproduction and offspring developmental deformities. After gathering information on the phenotypes produced after BaP exposure (Corrales et al.

2014), continued work will hopefully determine the molecular mechanisms to develop an adverse outcome pathway for the effects of BaP.

MATERIALS AND METHODS

2.1 Fish source, care and handling

AB line wild-type zebrafish were purchased from Zebrafish International Resource Center (ZFIN, Eugene, OR) and raised under the approved IACUC protocol. Fish were kept in Aquatic Habitats ZF0601 Zebrafish Stand-Alone System (Aquatic Habitats, Apopka, FL) with zebrafish water (pH 7.0-7.5, 60 parts per million (ppm), Instant Ocean, Cincinnati, OH) at 25-28°C, 14:10 light-dark cycle. Fish were fed twice daily with TetraMin® Tropical Flakes and live brine shrimp. Sexually mature fish without any deformities or signs of disease were selected as breeders. Their eggs were collected and larvae were raised to 120 days post fertilization (dpf) to obtain the F0 generation for the dietary exposure described below.

2.2 BaP flake concentrations

To prepare the treated flake food, 24 g of flake food were spiked with 18 ml of acetone containing BaP (0, 0.01667, 0.1667, or 1.667 µg/L). This was equivalent to nominal BaP concentrations in the food of 0, 12.5, 125, or 1250 µg/g food (control, low, medium, and high dose groups respectively). Treated flake food was extracted with methylene chloride to confirm the nominal concentrations of BaP. Approximately 10 mg of flakes were extracted right before day 0 and on days 7 and 14 with 2-3 mL of

methylene chloride. A known concentration of a surrogate standard, benzo[a]pyrene-d12, was added to each sample to yield a final concentration of 0.2 µg/mL. Samples were vortexed for 30 seconds and centrifuged for 7 minutes at 2000 rpm (668 x g). Samples were then blown to dryness with N₂ and brought back up with a known volume of hexane. A known concentration of internal standard, fluorene-d10, was added to each sample to determine extraction efficiency. Samples were run on the GC/MS under the selected ion mode to quantitate the concentration of BaP in each sample. Percent recoveries ranged from 70 to 145%.

2.3 BaP dietary parental (F0) exposure

Adult zebrafish (120 dpf) in the F0 generation were fed food treated with either acetone or 0.25, 2.5, or 25 µg/g fish nominally equivalent to 12.5, 125, or 1250 µg/g food. Fish were fed at a feed rate of 1% body weight twice/day for 20 and 21 days before collecting eggs. Acetone was used as the BaP solvent because previous findings in our lab showed feed rate and egg production did not vary between fish fed acetone-treated and non-treated food. Acetone was purchased from Fisher Scientific (Fair Lawn, NJ) and BaP from Supelco Analytical (Belfonte, PA). The spiked flakes were immediately rotavapped to dryness and stored in amber vials at room temperature. Each treatment group contained 11 tanks with four fish (two male and two female) per tank. The fish were fed 1% of their body weight in dosed flakes twice a day and given live brine shrimp once a day. Water in the tanks was changed every other day.

2.4 Reproductive success of F0 generation

Eggs from the F0 generation were collected on days 20 and 21 of the exposure using weighted egg collection baskets. These eggs were sorted and total number of eggs and number of fertilized eggs were recorded. A subset of 45 fertilized eggs was placed in 12-well plates (5 embryos/well containing 4 ml of 0.05% methylene blue). This generated a subset of 450 embryos per group. The eggs were continually monitored at 24, 32, 48, 56, 72, 80, and 96 hours post fertilization (hpf) and both survival and hatching rates were recorded. At 96 hours, a subset of 50 larvae per group was examined and photographed for morphological abnormalities. These abnormalities included body length and shape, tail shape, pectoral fins, craniofacial features (optic vesicle, otic vesicles, jaws, fore-, mid-, and hindbrain), pericardial edema, swim bladder shape and size, and yolk sac edema.

2.5 Reproductive success of F1 and F2 generations

Conditions and procedures outlined above for the F0 generation were used to raise the selected F1 and F2 fish. At maturity, F1 fish were allowed to acclimate for at least one week while maintained at 25.5-28°C and fed twice daily with TetraMin® Tropical Flakes and live brine shrimp. On days eleven, twelve, and thirteen, eggs were collected from F1 fish that were then raised for the F2 generation. F2 fish were also allowed to grow to maturity and then acclimate to the same conditions. On days 25 and 26, eggs were collected from F2 fish that were raised for the F3 generation. For each egg collection, eggs were collected from each tank twice. Incidences of survival, hatching, and morphological abnormalities were recorded for all larvae from each generation.

2.6 Histopathology of BaP effects on gonads

Fish were euthanized and a midventral incision through the abdominal body wall was made to facilitate fixation of the internal organs. Fish were placed in Dietrich's fixative (30% ethanol, 10% formaldehyde, 2% acetic acid) for two weeks at room temperature shaking, decalcified in 10% formic acid, dehydrated in graded ethanol series (70-100%), cleared in Clearify, incubated in paraffin for 24 h, and embedded in paraffin. Tissue blocks were sectioned using an Olympus Cut 4055 microtome at 8 μ m, mounted on glass slides and stained with hematoxylin and eosin (H&E). Photographs were taken with a MicroFire camera and PictureFrame 2.3 software (Optronics®, Goleta, CA) attached to an Olympus BX40 microscope.

2.7 Scoring of F0 ovaries

Ovaries of the F0 generation were sectioned using the technique outlined above and examined using NIH Image J. Using classifications outlined in *Histological Analysis of Endocrine Disruption Effects in Small Laboratory Fish* by Daniel Dietrich and Heiko O. Krieger (ISBN:978-0-471-76358-1) pre-vitellogenic, vitellogenic, mature, and corpus atreticum oocytes were quantitated and expressed as a percent. Percentages were calculated as (number of classified oocytes/total oocytes) x 100%.

2.8 Statistical analysis

Results were analyzed using GraphPad Prism 5.0 (La Jolla, CA) and presented as mean \pm S.E. Data sets were first analyzed by the Kolmogorov-Smirnov test to determine if they were normally distributed. Mortality, hatching, and ordinal data of developmental

deformities were analyzed using the 1-way ANOVA followed by Tukey's post hoc test or using Kruskal-Wallis followed by Dunn's post hoc test if the Kolmogorov-Smirnov test failed. Deformity incidence across score classifications was analyzed by 2-way ANOVA. Statistical significance was accepted at $p \leq 0.05$ for all tests.

RESULTS

3.1 BaP flake concentrations

BaP was not identified in the acetone-treated samples. Actual BaP concentrations of the treated flakes were: 10.44 ± 0.4 , 113.61 ± 2.3 , and $1012 \pm 30.7 \mu\text{g BaP/g flake}$ equivalent to 0.209 ± 0.008 , 2.27 ± 0.046 , and $20.25 \pm 0.614 \mu\text{g BaP/g fish}$, respectively.

3.2 Egg production and fertilization success

The fish in the F0 generation who were exposed to the highest dose of BaP had significantly lower number of fertilized eggs produced as compared to the controls. While there was a downward trend, there was no significant difference among treatment groups and control group of total eggs produced per tank. There was also no significant difference among treatment groups for fertilization success when expressed as a percent (Figure 2).

3.3 F0 gonad staging

Staging of F0 ovaries showed no significant difference in the percentage of previtellogenic oocytes, vitellogenic oocytes, or mature oocytes (Figure 3 and Table1).

There was a significant decrease in the percent of corpus atreticum in the fish fed 20 µg BaP/g as compared to controls. Staging of F0 testes examined percent cyst surface area of spermatogonia, spermatocytes, spermatids, and spermatozoa and found no significant difference between control and high dose BaP (Figure 4 and Table 2).

3.4 Hatching percent of larvae

In the F1 generation, more fish hatched earlier (at 48 hpf) in the offspring of F0 fish who were exposed to high doses of BaP. This was represented as a significantly higher percent of embryos that hatched before this time point. No other groups in the F1 generation showed any significant difference in percent hatched. There was also no significant difference among any groups in the F2 or F3 generations (Figure 5).

3.5 Cumulative mortality of larvae

In the F1 generation, by 48 and 56 hpf larvae from F0 parents in both the medium and high dose BaP groups had significantly higher mortality (55.2% at 48 hpf and 57.7% at 56 hpf) as compared to larvae from the control group (~27% at 48 hpf and 56 hpf). There was no significant difference from the control group in the larvae from the fish exposed to low doses of BaP. In the F2 and F3 generations, there was no significant difference in larvae mortality in any group (Figure 6).

3.6 Morphological deformities in larvae

The F1 generation offspring of parents who were exposed to medium dose BaP had significantly fewer fish classified as normal body shape as compared to control and low

dose F1 fish. Similar results were seen in the F2 generation where fish from medium and high dose groups had fewer normal body shape fish. Body length was also significantly decreased in F1 fish from parents exposed to medium and high doses of BaP as compared to controls (Figures 7 and 8). In the F2 generation fish from F0 predecessors exposed to high doses of BaP, there were also significantly fewer brains classified as normal as compared to F2 fish from control predecessors (Figure 9).

DISCUSSION

A parental (F0) BaP dietary exposure in zebrafish proved to be a successful model to evaluate subsequent multigenerational deformities in offspring (F1, F2, and F3 generations) that were never fed a BaP-treated diet. PAHs are taken up by aquatic organisms from the water or through the diet (Hylland 2006). The human relevance of this dietary exposure to PAHs is based on the high levels of the PAH BaP in human diet and the negative impact BaP exposure has on both mother and fetus. However, controlled laboratory dietary BaP exposures in fish are scarce. For example, zebrafish were fed twice a day 0, 19, or 110 μg BaP/g diet for 260 days as part of a larger experiment to investigate effects on retinoids and reproduction (Alsop et al., 2007). In Alsop et al.'s study, BaP did not affect reproduction (number of eggs produced or fertilization success), and no deformities were observed in 36 hpf embryos. Inconsistencies in results between Alsop et al.'s experiment and the work in this study can be explained by fundamental differences in experimental design: for example, here fish were fed 10, 114, or 1012 μg BaP/g diet at a feed rate of 1% body weight twice/day for 20 days before collecting eggs; by contrast, they fed fish up to 110 μg BaP/g diet for 245 days before collecting eggs.

Moreover, they evaluated developmental deformities by “visual inspection” at 36 hpf at which time point development (organogenesis) is still occurring. Other dietary BaP exposures in fish have been done, but none have examined its effects on development and reproduction (Couillard et al. 2009, Roesijadi et al., 2009, Yuen and Au 2006). Therefore, dietary BaP effects on the development of anatomical structures in offspring remain unknown in most species when considering the effects on F2 and F3 generations.

Most previous embryonic studies in fish have utilized waterborne exposures to mixtures of PAHs. That is, embryos were directly exposed to PAHs (Garner and Di Giulio 2012; Incardona et al., 2011; Carls and Meador 2009; Barron et al., 2004; Colavecchia et al., 2004; Kocan et al., 1996). Here embryos were not directly exposed, and BaP was not detected in F1 embryos after parents were fed a BaP-treated diet for 21 days indicating that there was potentially no transfer of BaP directly from mother to embryo. Alternatively, if BaP was rapidly metabolized in the mothers, BaP metabolites could have been deposited in the embryos. In a *Fundulus heteroclitus* study, ten embryos were pooled to determine BaP and BaP metabolite concentrations, and while BaP and BaP 9,10-dihydrodiol were recovered, all of the other metabolites (BaP-7,8,9,10-tetrahydrotetrol, BaP-7,8-dihydrodiol, BaP-1,6-dione, BaP-3,6-dione, BaP-6,12-dione, BaP-9OH and BaP-3-OH) remained below detection limits (Wills et al., 2009). Zebrafish embryos are smaller than *Fundulus heteroclitus* embryos, and therefore more than ten times as many zebrafish embryos were used to extract BaP albeit undetectable in the present study. BaP metabolites were not extracted, and conclusions cannot be drawn about their direct interaction. Despite no detectable BaP in the embryos, there was still significant mortality in F1 offspring of exposed parents. Thus, reasons for the increased

F1 mortality could be due to effects on parental reproduction or direct embryonic toxicity of BaP metabolites.

BaP is a known carcinogen as well as an endocrine disrupter. Both male and female fertility and reproductive ability are affected by exposure to PAHs. Airborne exposure studies have shown reduced sperm progressive, or forward, motility as well as decreased testosterone levels in male rats (Inyang et al. 2003). Other studies indicated negative multigenerational effects on several aspects of male fertility in mice after BaP exposure (Mohamed et al., 2010). These studies speculated several potential mechanisms of the negative impacts of BaP on male fertility. Reactive oxygen species (ROS) are found in the testes after BaP exposure and are thought to damage sperm by oxidative attack on the plasma membranes (Inyang et al. 2003). Metabolites of BaP also accumulate in the testes and significantly decrease the number of Leydig cells which decreases circulating testosterone levels (Inyang et al. 2003). Other proposed mechanisms include epigenetic effects such as altered DNA methylation that could explain the multigenerational effects (Mohamed et al., 2010).

There also has been evidence of negative impacts on female reproduction and ovotoxicity in exposures to BaP in mice (Sobinoff et al., 2012). Sobinoff et al.'s study found both oocyte and female gonad damage after exposure to BaP (2012). Similar to Inyang et al.'s study on sperm, the hypothesized mechanism of damage to oocytes involved oxidative stress and the production of ROS that decreased sperm-egg binding ability (Sobinoff et al., 2012). Sobinoff et al.'s study also found that BaP induced up-regulation of several genes that cause premature follicle activation and increased developing follicle atresia (2012). One perplexing aspect of our study involves our

findings of reduced percentages of atresia in the ovaries of fish exposed to BaP as compared to controls. This is the opposite of what we would expect due to findings that BaP induces the metabolism of estrogen that is also a hypothesized method of premature follicular activation (Sobinoff et al., 2012). However, other studies have shown that concentrations of BaP similar to estimated concentrations found in women who smoke decrease overall follicle stimulation in cultured rat follicles exposed to BaP (Neal et al., 2007). More work should be done to determine the true mechanism of the negative effect of BaP on female fertility.

Our study utilized zebrafish rather than a mammalian model to study the multigenerational effects on reproduction. This allowed us larger numbers of offspring to examine and a faster turnover time between generations. The fish exposed to the high dose of BaP demonstrated a statistically lower number of fertilized eggs produced per tank as compared to controls. While no other statistically significant results were found, there were differences in reproductive success among the groups. Further studies should be done to determine whether male fertility, female fertility, or both are the culprit for these differences.

Time to hatch after exposure to PAHs including BaP can either decrease (premature hatching) or increase (delayed hatching). It appears that the effect on time to hatch might be dose dependent. In our study, we found that the number of embryos that hatched early in the F1 high dose group was significantly higher (premature hatching) than the control and medium dose groups. This premature hatching can, in some ways, be equated to human premature birth. While the methods of hatching in fish are not like the methods of

human birth, BaP has been shown to cause decreased birth weight and length which are factors that are also associated with premature birth.

The negative impact on survival of fish embryos and larvae due to exposure to PAHs is well-documented in the literature. We found that mortality significantly increased in the F1 generation after parents (F0) had been fed medium and high doses of BaP. This result was consistent with previous findings that waterborne exposure to PAHs in embryos and larvae increased mortality (Carls and Thedinga, 2010; Barron et al., 2004; Hawkins et al., 2002; Carls et al., 1999). The same was true when zebrafish embryos were exposed to BaP alone (Fang et al., 2013; Bugiak et al., 2010). While we did not see a significant effect on mortality in the F2, F3, and F4 generations, 1 $\mu\text{g/L}$ BaP negatively affected the survival in F2 fathead minnow embryos after a waterborne exposure (White et al., 1999). We hypothesize that the reason for the lack of an effect on mortality in the F2, F3, and F4 generation is that only the fittest fish survived from the F1 generation, and thus F1's offspring survival was not negatively impacted.

Body morphology deformities such as body length, body shape, and brain abnormalities were the most severely impacted in two generations (F1 and F2). To our knowledge, this is the first report on a parental dietary exposure in fish still causing deformities at least two generations later. Now that multigenerational phenotypes are established, hypotheses can be made and tested as to the molecular pathways that are altered by BaP multigenerational exposure. Developmental deformities caused by BaP of the zebrafish larvae can be a model for developmental deformities seen in human offspring after maternal exposure to PAHs. Several studies have shown the connection between air pollution by PAHs, including BaP, and decreased birth weight, size, and

cognitive development in children born to mothers who are exposed (Choi et al., 2006; Perera et al., 2006; Perera et al., 2009; Tang et al., 2014). Our study provided evidence that a dietary exposure can not only negatively impact the offspring of those who are exposed, but also subsequent generations.

The principal result of this experiment, multigenerational adverse effects on developmental phenotypes and reproduction, invites reevaluating the priority of multigenerational studies which more closely reflect deleterious impacts on an ecosystem level versus single generation studies, which harbor organismal level impacts alone. Here we showed for the first time that a parental dietary exposure alone can cause deformities in fish two generations later. This indicates that fish can display deleterious effects due to harmful exposure only directly affecting their grandparents and great-grandparents. Future results of the dietary exposure in our group are directed to anchoring phenotypic deformities to genotypic changes such as changes in the transcriptome and methylome, which will allow us to develop adverse outcome pathways from the molecular level in an individual to phenotypic effects across multiple generations.

ACKNOWLEDGMENTS

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FIGURES

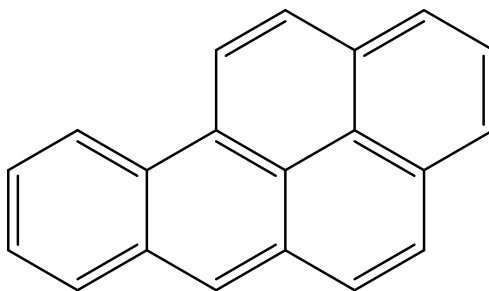


Figure 1. Benzo[a]pyrene (BaP). BaP is a polycyclic aromatic hydrocarbon.

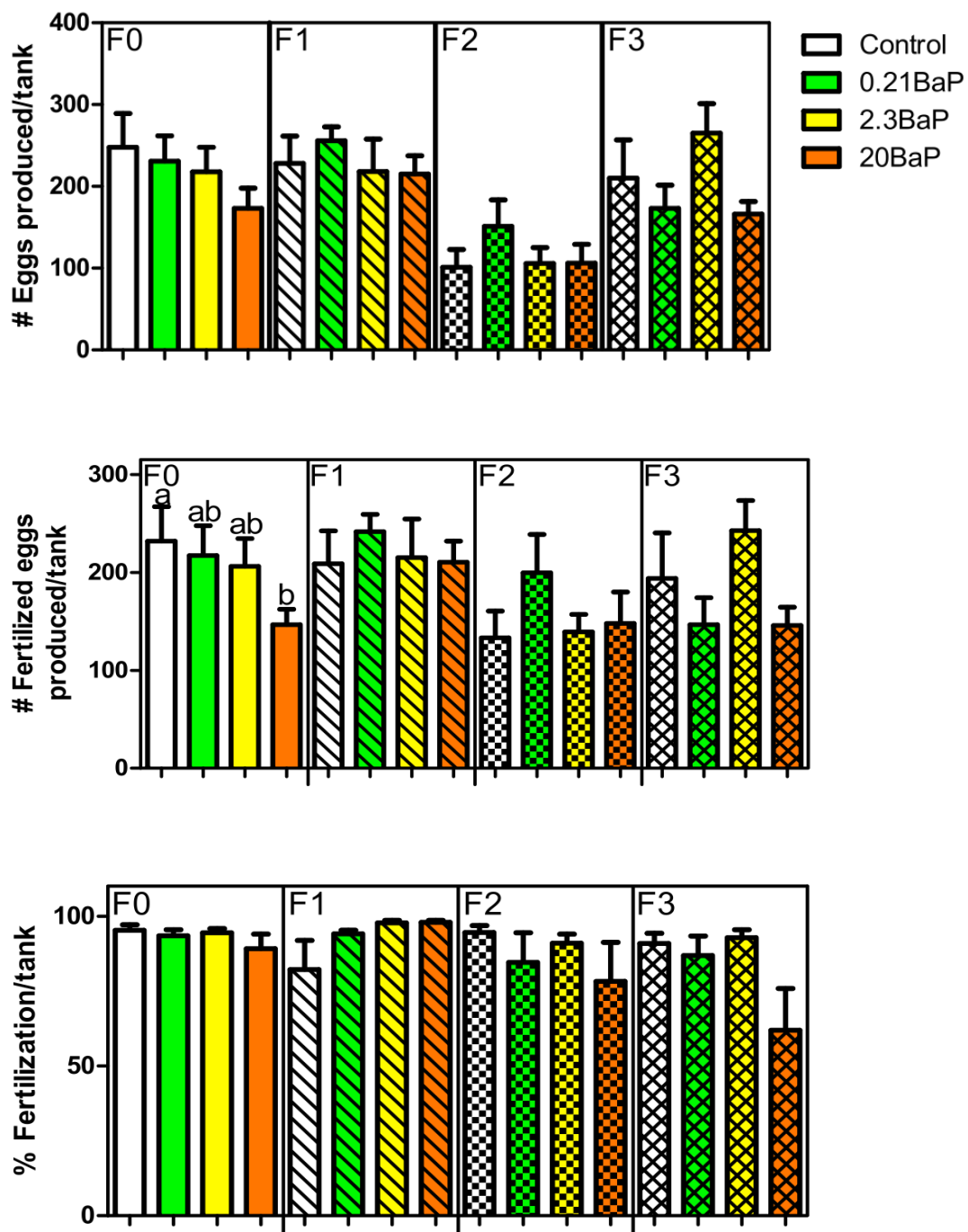


Figure 2. Egg production (A), # fertilized eggs (B) and % fertilization (C) of F0-F3 generations. Different letters represent statistical differences within a generation (n=10 tanks; ANOVA- Tukey post hoc test).

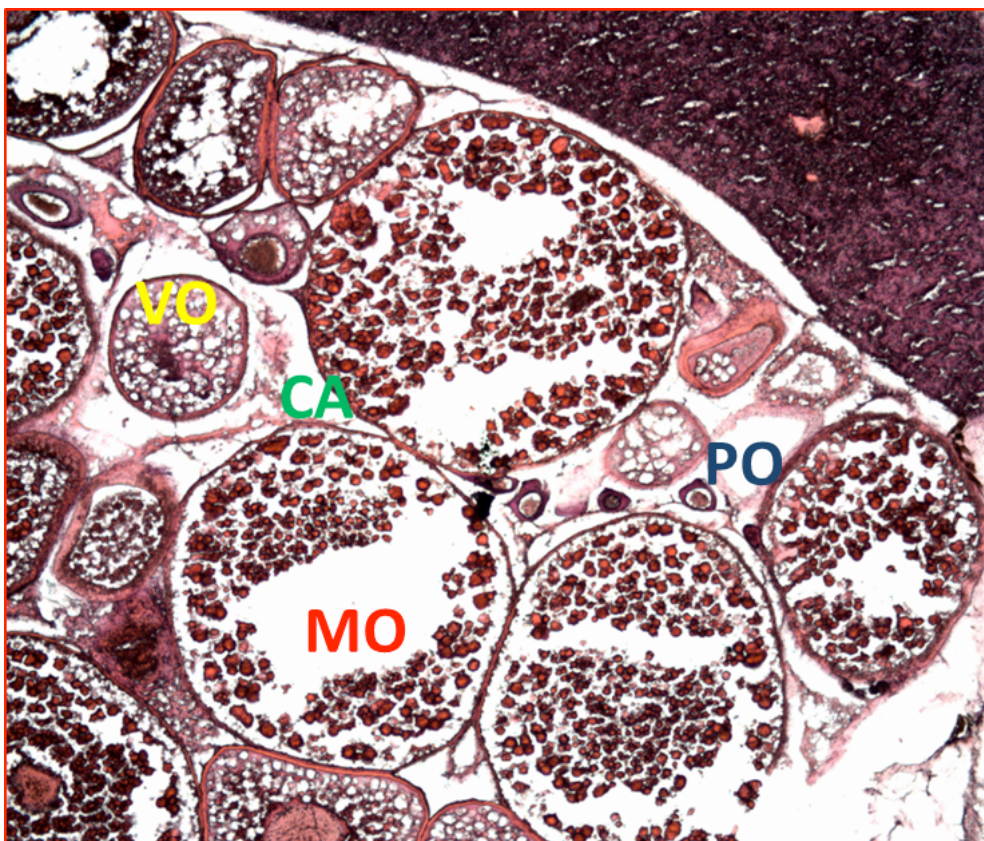


Figure 3. F0 oocyte staging. Sections from F0 female: PO=Previtellogenic Oocyte; VO=Vitellogenic Oocyte; MO=Mature Oocyte; CA=Corpus Atreticum. The percentage of corpus atreticum was significantly decreased in the fish exposed to high doses of BaP as compared to controls.

Table 1. F0 oocyte staging. Different letters within a column indicate significant differences. (n=5 fish; scored an average of 194 oocytes/fish; Kruskal-Wallis).

	% Pre-Vitellogenic	% Vitellogenic	% Mature	% Atresia
Control	54 ± 7 ^a	39 ± 6 ^a	4.3 ± 0.9 ^a	3.9 ± 1 ^a
0.21 BaP	61 ± 3 ^a	31 ± 2 ^a	5.1 ± 2.2 ^a	2.8 ± 0.9 ^{ab}
2.3 BaP	63 ± 5 ^a	31 ± 5 ^a	2.6 ± 0.9 ^a	2.5 ± 0.8 ^{ab}
20 BaP	56 ± 3 ^a	36 ± 3 ^a	7.1 ± 3.1 ^a	0.4 ± 0.2 ^b

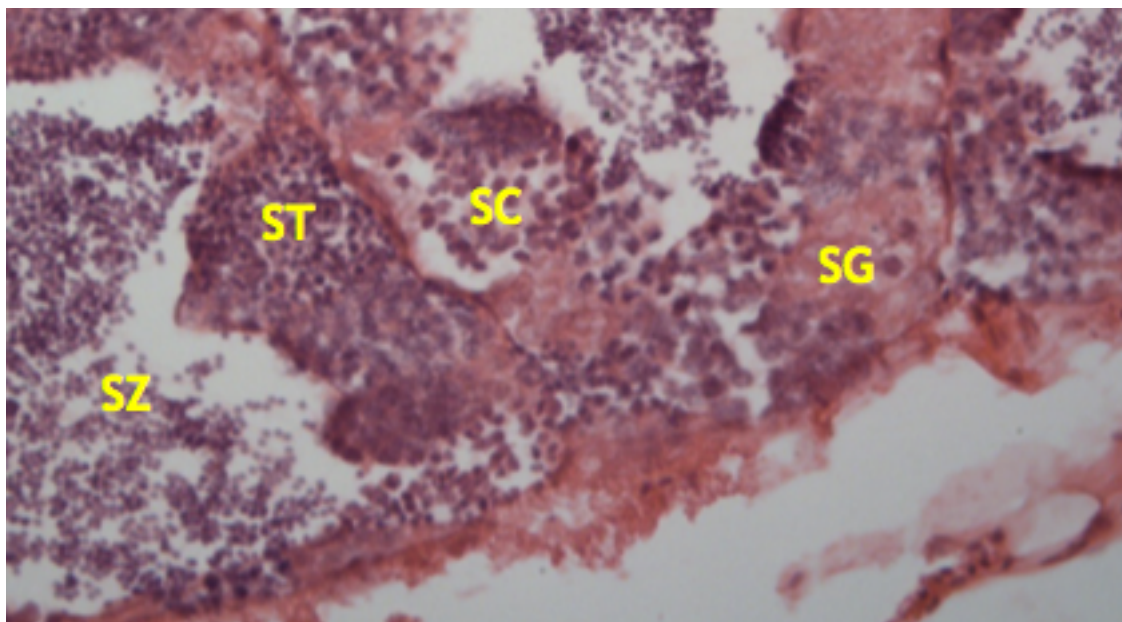


Figure 4. F0 testes staging. Average cyst size of SG=Spermatogonia, SC=Spermatocyte, ST=Spermatid, and SZ=Spermatozoa was not significantly altered in any dosing group as compared to control. The percentages were calculated as (# of cysts within a stage/total cysts observed) x 100%. (n = 5 per treatment; data was checked for normality with Kolmogorov-Smirnov test. If data passed normality, unpaired student t-test was used; if data was not normally distributed, Mann-Whitney test was used.)

Table 2. F0 testes staging. No statistical differences were found between the control and high dose groups.

	% Spermatogonia	% Spermatocytes	% Spermatids	% Spermatozoa
Control	23 ± 2 ^a	46 ± 4 ^a	19 ± 1 ^a	12 ± 3 ^a
20 BaP	27 ± 3 ^a	36 ± 6 ^a	22 ± 3 ^a	15 ± 2 ^a

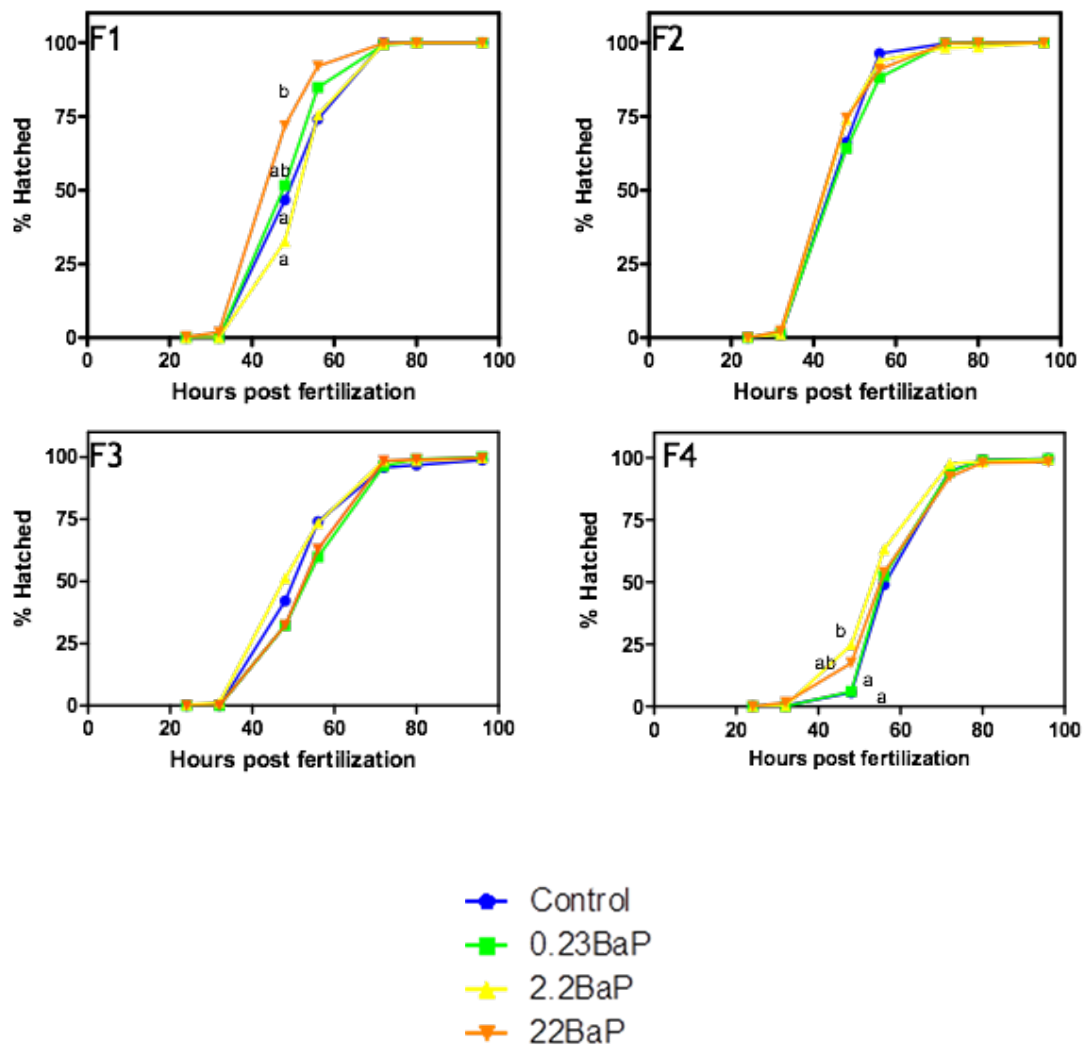


Figure 5. Percent hatched in F1, F2, F3, and F4 generations. Different letters represent statistical differences within a generation (n=10 tanks; ANOVA).

Cumulative Mortality

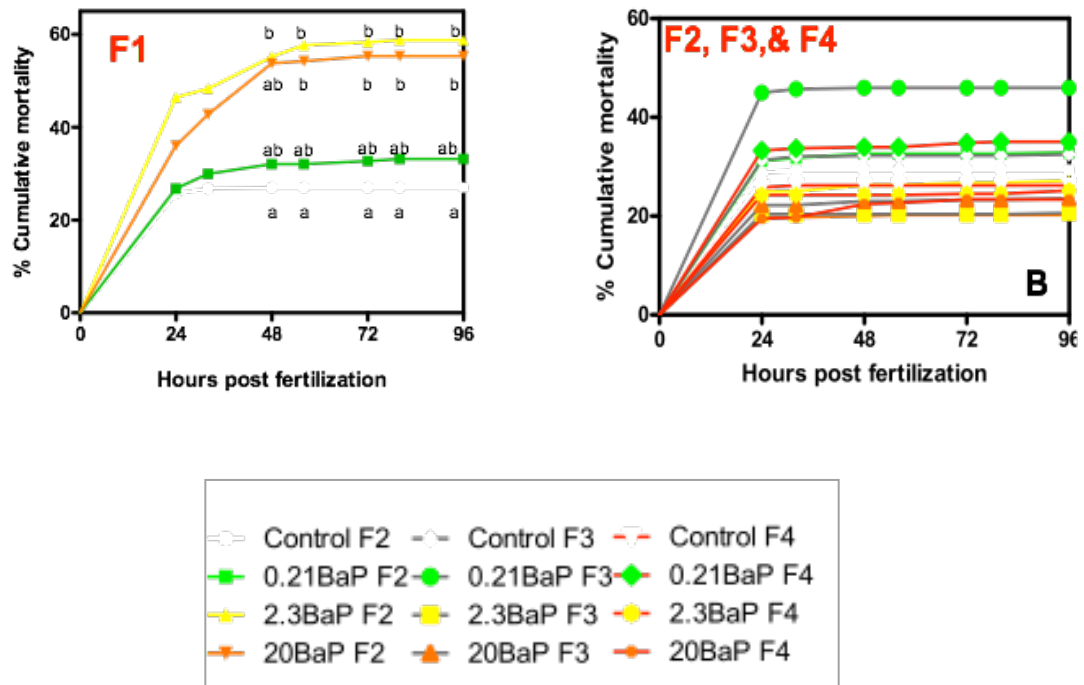


Figure 6. Cumulative mortality of F1, F2, F3 and F4 larvae. A) By 48 and 56 hpf, larvae whose parents were exposed to 2.3 and 20 μg BaP/g fish, respectively, had significantly higher mortality compared to control. B) No significant differences were observed in the F2, F3, and F4 generations. (ANOVA; n=8-10 tanks; 50 embryos/tank).

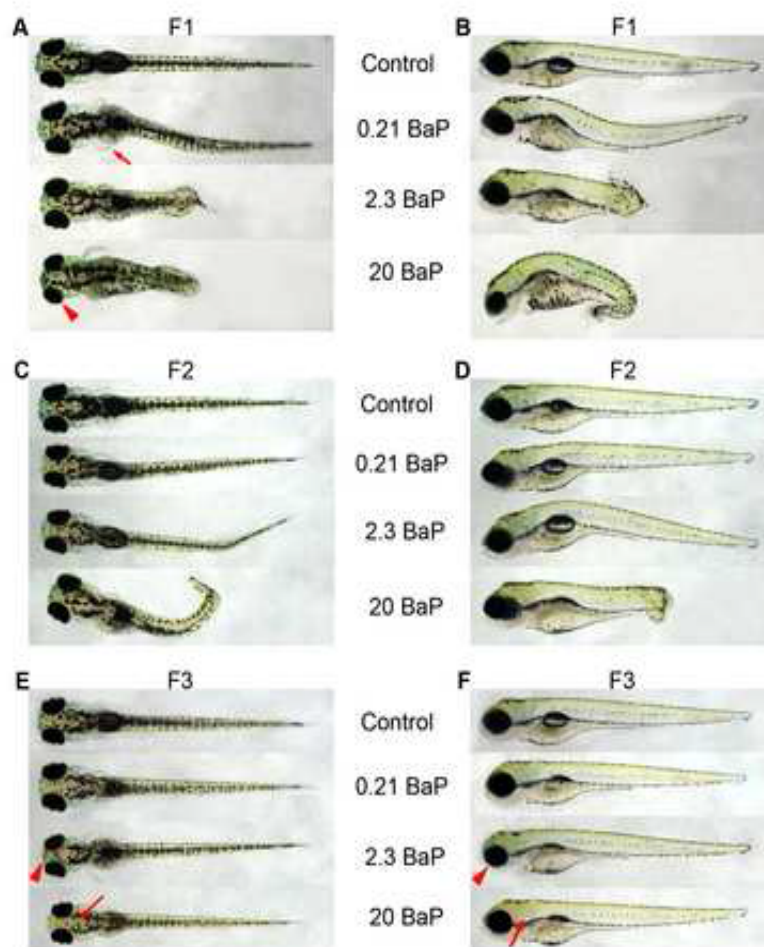
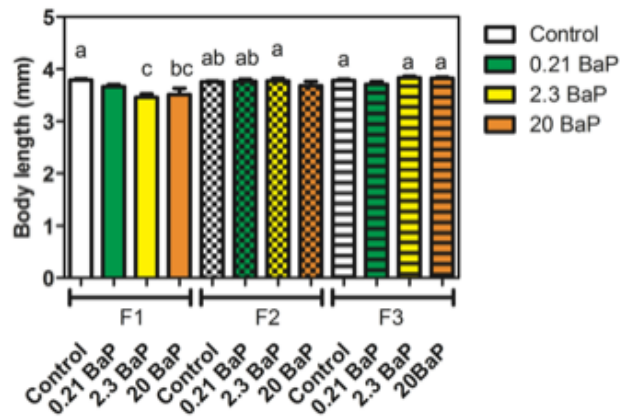


Figure 7. Representative dorsal (A, C, and E) and lateral (B, D, and F) view photos of 96 hpf larvae documenting developmental deformities across F1, F2, and F3 generations after a parental (F0) dietary exposure of BaP. Dorsal view photos were useful for evaluating pectoral fin deformities (A, red arrow 0.21 BaP). Body length and body shape defects were apparent in F1 and F2 generations (A, B, C, and D). Optic vesicle (eye) size (A, red arrowhead 20 BaP), brain size (E, red arrowhead), pigmentation (E, red arrow), absent jaw (F, red arrowhead), and absent otolith (F, red arrow) are examples of the deformities observed. N = 50 fish per treatment group.

A. Body Length



B. Body Shape

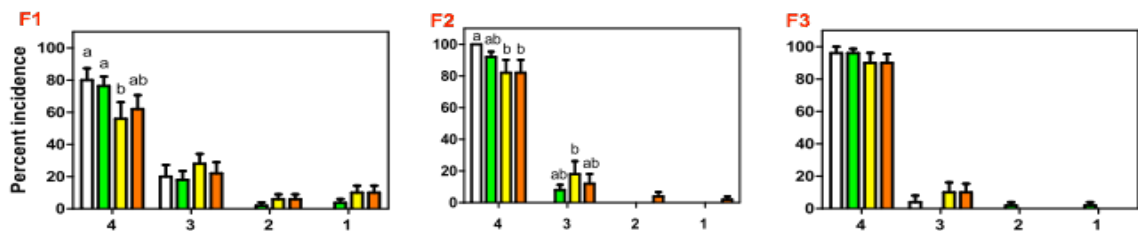


Figure 8. F1, F2, and F3 morphological abnormalities: A) body length and B) body shape. Body length was significantly reduced in the F1 generation following F0 exposure to 2.3 and 20 μg BaP/g fish. F0 exposure to 2.3 μg BaP/g fish significantly reduced the F1 and F2, but not F3, incidence of normal body shape (B). For body shape, 4= normal; 3=mild deformity; 2=moderate deformity; and 1=severe deformity. Different letters indicate significant differences and bars with no letters indicate no significant differences among any group (ANOVA; n=10 tanks; 50 larvae/tank).

Brain Deformities

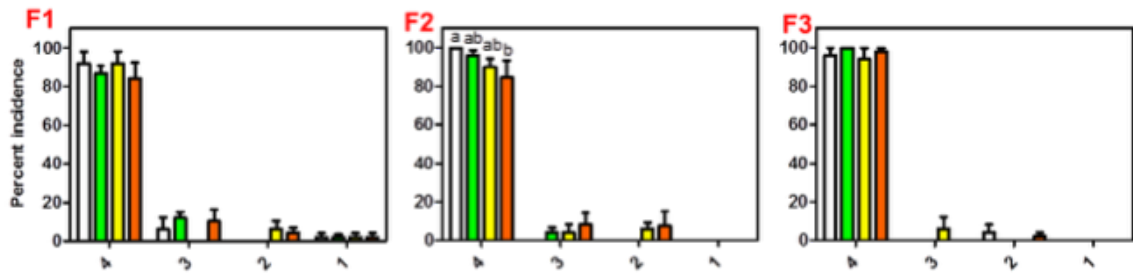


Figure 9. F1, F2, and F3 morphological abnormalities continued: Brain deformities included enlargement or reduction of the brain regions or absence of brain junctions. Score 4 = normal, 3 = mild deformity, 2 = moderate deformity, and 1 = severe deformity. N = 10 tanks per treatment group; 5 larvae per tank. Bars represent the mean \pm S.E. Different letters indicate significant differences between groups per score category (two-way ANOVA, $p < 0.05$).

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